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# Functional properties of *in vitro* excitatory cortical neurons derived from human pluripotent stem cells

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Key words: pluripotent, stem cell, cortical, neuron, function

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neurons and glia derived from pluripotent stem cells. **David Wyllie** is Professor of Ion Channel Physiology and Pharmacology and Director of the Centre for Integrative Physiology at The University of Edinburgh. Current research in his lab includes the use rodent models of monogenic causes of neurodevelopmental disorders to study the properties of altered synaptic function and to assess the extent to which pharmacological intervention can ameliorate the dysfunction that is observed in such models. This work is complemented by studies of defined neuronal and glial populations derived from human pluripotent stem cells and specifically those from individuals suffering from neurodevelopmental and neurodegenerative diseases.



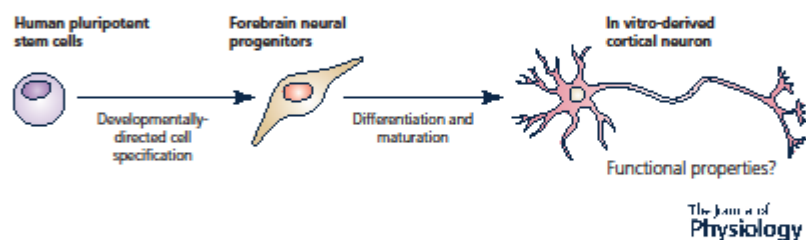


## ABSTRACT

The *in vitro* derivation of regionally defined human neuron types from patient-derived stem cells is now established as a resource to investigate human development and disease. Characterisation of such neurons initially focused on the expression of developmentally regulated transcription factors and neural markers, in conjunction with the development of protocols to direct and chart the fate of differentiated neurons. However, crucial to the understanding and exploitation of this technology is to determine the degree to which neurons recapitulate the key functional features exhibited by their native counterparts, essential for determining their usefulness in modelling human physiology and disease *in vitro*. Here, we review the emerging data concerning functional properties of human pluripotent stem cell-derived excitatory cortical neurons, both in the context of maturation and regional specificity.

## ABBREVIATIONS

hPSC, human pluripotent stem cell; hPSC<sup>C</sup> neuron, hPSC-derived excitatory cortical neuron; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; Shh, sonic hedgehog; RMP, resting membrane potential; Ligand-gated ion channel, LGIC;  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, AMPA; N-methyl-D-aspartate, NMDA;  $\gamma$ -aminobutyric acid type-A, GABA<sub>A</sub>; Strychnine-sensitive glycine receptor, GlyR.



## INTRODUCTION

The human cerebral cortex controls higher cognitive abilities including those that distinguish human beings from other mammalian species such as abstract thinking and complex language (Defelipe, 2011). This highly complex structure consists of several distinct areas that are responsible for processing and integrating different motor and sensory information



and information stored in memory. Further functional and anatomical sophistication is reflected in the diversity and number of excitatory and inhibitory cell types, each characterised by distinct gene expression, morphology and functional properties (Molyneaux *et al.*, 2007; Lui *et al.*, 2011). The cortex is a target for many disorders of the brain at all stages of life. For example, perturbation of cortical development can lead to neurodevelopmental disorders, such as autism spectrum disorders, while the adult cortex is a major site for certain neurodegenerative conditions including Alzheimer's disease, frontotemporal dementia and Huntington's disease.

The majority of studies investigating cortical development and neuronal function have been based upon rodent models, but several aspects of the human cortex differ greatly from rodents that includes tangential expansion in the frontal cortex and gyrencephaly (Defelipe, 2011; Lui *et al.*, 2011). Recently many laboratories have established *in vitro* protocols to derive excitatory pyramidal neurons, the principal neuronal type in the adult cortex, from human pluripotent stem cells (hPSCs) that provide powerful, readily accessible tools to model neuronal function in both healthy and disease contexts (Hansen *et al.*, 2011; van den Aamele *et al.*, 2014).

The ability of neurons derived from mouse and more recently human stem cell lines to exhibit classical neuronal functional properties both *in vitro* and when integrated into host systems has been studied for many years (Benninger *et al.*, 2003; Wernig *et al.*, 2004). Increasing refinement and control in the derivation of neurons generated from embryonic or induced pluripotent stem cells (ESCs/iPSCs) now means it is possible to derive regionally-specific neurons, including hPSC-derived excitatory cortical neurons (hereafter termed 'hPSC<sup>C</sup> neurons'). This raises the question as to the extent such cells are able to recapitulate known details of native cortical development and ultimately whether they are appropriate models of "diseases in a dish" (Sandoe & Eggan, 2013). In addition to forming synaptic connections to generate the intricate circuitry responsible for complex cortical processes, native excitatory cortical neurons undergo distinctive developmental changes in ion channel expression and ionic gradients that determine their function within cortical networks (Moody & Bosma, 2005). This review will principally focus on the emerging data examining the functional capability of hPSC<sup>C</sup> neurons to exhibit known native-like properties with regard to functional maturation and regional specification.

## SUMMARY OF CORTICAL DEVELOPMENT

The cerebral cortex organises in a complex 3-dimensional structure comprised of several anatomically distinct cortical areas. Cortical regionalization, within the developing telencephalon, is determined by graded expression of transcription factors expressed by cortical progenitor cells, including Gli3, Emx2, Pax6, Sp8 and Coup-TF1 (O'Leary *et al.*, 2007). This is in turn established by three major telencephalic signalling centres: the cortical hem that secretes Wnts and bone morphogenetic protein, the anterior neural ridge that secretes Fgf8 and the ventral telencephalon that secretes sonic hedgehog (Shh). Once the major cortical axes have been established, cortical progenitor cells generate neurons through the process of cortical lamination in a time-dependent manner (Gaspard & Vanderhaeghen, 2011). During this process, later-born neurons migrate over the early-born neurons within the cortical plate forming a layered structure in an inside-out fashion. Therefore, the deepest cortical layer VI forms first and the upper layer II last; the marginal zone or layer I, containing reelin<sup>+</sup> Cajal-Retzius cells, escapes this inside-out process (Frotscher, 1998). The adult cortex has a structure made up of 6 defined layers (I-VI) of diverse excitatory cortical neuron types that can be identified by layer-specific markers, including CUX1 (layer II/III), ROR- $\beta$  (layer IV), CTIP2 (layer V), SOX5 (layers VI and V) and TBR1 (layer VI) (Molyneaux *et al.*, 2007).

The correct time-dependent patterning of the telencephalon and the formation of different neuronal subtypes within the different layers are ultimately essential for the appropriate functional wiring of cortical neurons with other cortical or subcortical targets (Germain *et al.*, 2013). While neocortical excitatory pyramidal neurons form from distinct populations of cortical progenitor cells within the embryonic dorsal telencephalon, the majority of inhibitory interneurons originate in the ventral telencephalon and subsequently tangentially migrate into the developing cortex (Hansen *et al.*, 2013).

## SPECIFICATION OF IN VITRO hPSC<sup>C</sup> CULTURES

*In vitro*-derived cortical progenitor cells giving rise to hPSC<sup>C</sup> neurons can be formed from the spontaneous neuralisation of hPSCs (Eiraku *et al.*, 2008; Li *et al.*, 2009; Mariani *et al.*, 2012; Shi *et al.*, 2012) and neuralisation can be accelerated by using inhibitors of WNT and bone morphogenetic protein/NODAL signalling pathways (dual-SMAD inhibition; (Chambers *et al.*, 2009)). Moreover, the presence of Shh or Shh agonists can give rise to ventral telencephalic progenitors from which cortical interneurons can differentiate (Germain *et al.*, 2013; Maroof *et al.*, 2013). Furthermore, hPSC-derived cortical progenitors generally acquire a caudal identity by default as shown by their pattern of projections when transplanted in mouse brains (Espuny-Camacho *et al.*, 2013), yet they can be patterned to different cortical regions

and respond to signalling cues when treated with morphogen agonists (Eiraku *et al.*, 2008; Espuny-Camacho *et al.*, 2013; Kadoshima *et al.*, 2013). Moreover, whilst the temporal generation of neurons belonging to different layers is largely maintained *in vitro* and the presence of neurons belonging to all six layers has been reported, the contribution of each layer considerably varies depending on the method used (see (van den Aamele *et al.*, 2014)).

In these respects, *in vitro* neuronal connections and circuitry will be therefore somewhat limited by the protocol to recapitulate cortical development in its cellular specification and organisation, particularly in monolayer cultures. However, aspects of cortical cytoarchitecture are remarkably maintained *in vitro* in particular in 3-dimensional cultures that allow the radial localization of later-born neurons above earlier-born ones (Mariani *et al.*, 2012; Lancaster *et al.*, 2013; Paşca *et al.*, 2015).

Transcriptome analyses of the sequential phases of *in vitro* hPSC-derived corticogenesis in monolayer or 3D culture demonstrate the ability of these protocols to reproduce gene expression profiles in hPSC<sup>C</sup> neuron populations that are equivalent to early native human embryonic cortical development (Stein *et al.*, 2014; Paşca *et al.*, 2015). Importantly, the heterogeneity in the cellular specification of previously published protocols is also matched at the transcriptome level, where some protocols better recapitulate native development than others and, furthermore, have variable rates of hPSC<sup>C</sup> neuron maturation (Stein *et al.*, 2014). This data does not however directly examine the functional capacity of hPSC<sup>C</sup> neurons. Many groups therefore apply patch-clamp electrophysiology (and also live-imaging) to assess directly the functional membrane properties of hPSC<sup>C</sup> neurons.

## **hPSC<sup>C</sup> NEURONS ARE FUNCTIONALLY REMINISCENT OF IMMATURE CORTICAL NEURONS**

Neurons are defined by their excitable plasma membrane properties, which rely on the development of ionic gradients and the expression of ion channels. Reports demonstrating the generation of hPSC<sup>C</sup> neurons have therefore focused upon basic intrinsic membrane properties and the ability of hPSC<sup>C</sup> neurons to generate action potentials. Data indicate that intrinsic and action potential firing properties of hPSC<sup>C</sup> neurons are broadly comparable to rodent cortical neuron at an embryonic/early postnatal stage of development (Johnson *et al.*, 2007; Kim *et al.*, 2011; Espuny-Camacho *et al.*, 2013; Bilican *et al.*, 2014). Indeed, the

majority of studies of hPSC-derived neurons which have recorded electrophysiological properties consistently report input resistances that are 5- to 20-fold higher than their adult, mature *in vivo* counterparts (Johnson *et al.*, 2007; Kim *et al.*, 2011; Espuny-Camacho *et al.*, 2013; Bilican *et al.*, 2014). This is also true for hPSC-derived neurons cultured for extended periods. Thus, hPSC-derived neurons typically require only modest current injection to elicit action potential firing. hPSC<sup>C</sup> neurons that show increases in excitability over time also demonstrate maturation in their expression of intrinsic membrane conductances that collectively define the nature of neuronal excitability (Johnson *et al.*, 2007; Bilican *et al.*, 2014). Additionally, interneuronal content is very low in some *in vitro* cultures (Shi *et al.*, 2012; Bilican *et al.*, 2014), however many protocols do not report the extensiveness of interneuron differentiation in their cultures. It is important to note that interneuronal classes have highly variable mature firing properties (Markram *et al.*, 2004) and may resemble those of immature excitatory neurons, particularly in that they are also likely to be immature.

Studies have revealed that the resting membrane potential (RMP) of hPSC<sup>C</sup> neurons hyperpolarises with extended culture periods (Johnson *et al.*, 2007; Bilican *et al.*, 2014). Nevertheless RMPs can remain relatively depolarised and as such this compromises their ability to display spontaneous (TTX-sensitive) action potential firing which is considered critical to the development and maturation of the cortex (Spitzer, 2006). Indeed, (Weick *et al.*, 2009), demonstrated that TTX only weakly blocked spontaneous Ca<sup>2+</sup> transients in hPSC<sup>C</sup> neuron cultures indicating that these transients were largely not mediated by spontaneous action potential activity. Careful pharmacological work determined the source of Ca<sup>2+</sup> to be mediated through L-type voltage-gated Ca<sup>2+</sup> channels and transient receptor potential channels.

## LIGAND-GATED ION CHANNELS

Ligand-gated ion channels (LGICs) are integral to the process of fast neurotransmitter signalling and their activities contribute to the fine balance of excitation and inhibition within the CNS. LGICs are multimeric protein complexes that can be comprised of numerous subunit combinations which impose distinct biophysical and pharmacological properties. LGIC subunit composition is often regulated both developmentally and spatially. Thus, the assessment of LGIC composition in hPSC<sup>C</sup> neurons is essential if we are to determine the extent to which these cells reflect native properties.

## IONOTROPIC GLUTAMATE RECEPTORS

Ionotropic glutamate receptors are the central mediators of fast excitatory neurotransmission in the cortex and are a family of three tetrameric receptor types; AMPA, NMDA and kainate receptors (Traynelis *et al.*, 2010). NMDA receptors (NMDARs) are composed of two ubiquitously expressed GluN1 subunits and two potential GluN2A, GluN2B, GluN2C, GluN2D and/or GluN3A, GluN3B subunits (Wyllie *et al.*, 2013). Considerable evidence shows that NMDARs in embryonic mammalian cortical neurons contain predominantly GluN1 and GluN2B subunits while maturation is associated with a functional up-regulation of GluN2A subunits (Wyllie *et al.*, 2013). For rodents this is a postnatal event, however determining this in humans has proved challenging (Henson *et al.*, 2008). hPSC<sup>C</sup> neurons maintained in culture for 5 weeks express GluN1/GluN2B NMDARs as assessed by their sensitivity to the GluN2B-selective antagonist, ifenprodil and therefore an immature NMDAR profile (Livesey *et al.*, 2013).

AMPA receptors (AMPA<sup>R</sup>s) can be composed of GluA1, GluA2, GluA3 and GluA4 subunits of which the functional up-regulation of the GluA2 subunit is associated with cortical neuronal maturation (Traynelis *et al.*, 2010). GluA2 subunits undergo post-transcriptional modification resulting in an arginine codon [GluA2(R)] replacing a glutamine codon (GluA2(Q)) in the M2 pore-forming region of the channel. Therefore native cortical maturation is associated with a shift from GluA2(R)-lacking to GluA2(R)-containing AMPARs. Importantly, the presence of one or more GluA2(R) subunit in an AMPAR complex results in reduced single-channel conductance, reduced sensitivity to channel-blocking polyamines and, crucially, reduced Ca<sup>2+</sup>-permeability (Traynelis *et al.*, 2010). Assessment of the functional AMPAR composition in hPSC<sup>C</sup> neurons using non-stationary fluctuation analysis to estimate mean AMPAR single-channel conductance and their sensitivity to a GluA2(R)-lacking AMPAR channel blocker indicates an activity-independent and native-like maturation from GluA2(R)-lacking to GluA2(R)-containing AMPARs within 5 weeks of *in vitro* differentiation (Livesey *et al.*, 2014). GluA2 transcript expression also increases with time in culture (Chander & Weick, 2014) (Stein *et al.*, 2014; van de Leemput *et al.*, 2014). Thus AMPAR expression in hPSC<sup>C</sup> neurons appears to display properties that are observed in native mature neuronal populations (Isaac *et al.*, 2007). Both NMDARs and AMPARs are expected to undergo maturational changes in composition in the early postnatal weeks of cortical development in rodents (Traynelis *et al.*, 2010) and in this regard the ontogenetic development of AMPARs in hPSC<sup>C</sup> neurons is much more rapid than expected. Interestingly, the GluA2 subunit has been shown to be rapidly edited and functionally up-regulated (4 weeks) after the *in vitro* differentiation of neurons from primary human cortical progenitors

(Whitney *et al.*, 2008) in contrast to the expected longer *in vivo* developmental time scales (Talós *et al.*, 2006).

These data suggest the rapid maturation of the AMPAR complex is a potential product of the *in vitro* environment. Nonetheless, this feature provides an opportunity to examine numerous scenarios in which abnormal regulation of the GluA2 subunit is hypothesized or prevalent in adult human disease (Wright & Vissel, 2012).

## IONOTROPIC GABA AND GLYCINE RECEPTORS

GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) and strychnine-sensitive glycine receptors (GlyRs) are pentameric LGICs that primarily mediate fast inhibitory neurotransmission in the mature cortex. GABA<sub>A</sub>Rs can be potentially composed of 19 known subunits ( $\alpha$ 1–6,  $\beta$ 1–3,  $\gamma$ 1–3,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$  and  $\rho$ 1–3) giving a vast number of theoretical possible GABA<sub>A</sub>R arrangements. Whilst in reality composition is tightly regulated, the breadth of possible GABA<sub>A</sub>Rs composition generates considerable functional and pharmacological diversity across brain regions and cellular locations (Olsen & Sieghart, 2009). Using a pharmacological and RNA-seq based approach hPSC<sup>C</sup> neurons differentiated for 5 weeks were shown to express GABA<sub>A</sub>Rs that had a predominant  $\alpha$ 2/3 $\beta$ 3 $\gamma$ 2 composition (James *et al.*, 2014). This is the most common GABA<sub>A</sub>R combination present in the embryonic cortex (Olsen & Sieghart, 2009). Comparison of GABA<sub>A</sub>R subunit transcript levels in hPSC<sup>C</sup> neurons with data from human primary tissue indicates that overall GABA<sub>A</sub>R subunit expression is similar to that seen in the cortex at 12–21 weeks post conception. Again this is in broad agreement with other transcriptome based studies (Stein *et al.*, 2014; Paşca *et al.*, 2015). Finally, pharmacological assessment of GABA<sub>A</sub>Rs in hPSC<sup>C</sup> neurons is consistent with the absence of the  $\alpha$ 1-subunit which is associated with more developmentally mature cortical neurons.

GlyRs are thought to play an important role in cortical development and transient functional GlyR expression is a feature of neocortical development in rodents (Flint *et al.*, 1998; Avila *et al.*, 2013). Indeed, hPSC<sup>C</sup> neurons respond robustly to glycine application (James *et al.*, 2014). Pharmacological and RNA-seq analysis of GlyRs, indicates that the GlyR composition is principally  $\alpha$ 2/ $\beta$ -containing (James *et al.*, 2014). Transcript levels indicate a level of maturity equal to that of GABA<sub>A</sub>Rs, however it is thought that the early mammalian embryonic GlyR composition consists of homomeric  $\alpha$ 2 GlyRs and matures to  $\alpha$ 1/ $\beta$ -containing GlyRs (Lynch, 2009). Interestingly, a transient GlyR population of  $\alpha$ 2/ $\beta$ -containing

GlyRs have been observed in developing rodent Cajal-Retzius cells (Okabe *et al.*, 2004). hPSC<sup>C</sup> neurons may prove useful in elucidating the role of GlyRs within the developing human cortex.

## INTRACELLULAR CHLORIDE

GABA<sub>A</sub>Rs and GlyRs are permeable to Cl<sup>-</sup> ions and mediate inhibitory responses in adult cortical neurons. However, the application of either GABA or glycine to embryonic excitatory cortical neurons generates depolarising excitatory responses due to elevated levels of intracellular Cl<sup>-</sup>. (Ben-Ari *et al.*, 2007) The developmental reduction in intracellular Cl<sup>-</sup> concentration is a crucial feature of cortical development and its perturbation is implicated in numerous disease mechanisms (Blaesse *et al.*, 2009).

Intracellular Cl<sup>-</sup> activity in hPSC<sup>C</sup> neurons (and neural precursor cells) measured using the perforated patch-clamp technique falls from around 25 mM after 7 weeks in culture to <7 mM in hPSC<sup>C</sup> neurons (Livesey *et al.*, 2014). Correspondingly, the expression of K<sup>+</sup>-Cl<sup>-</sup> cotransporter-2 (responsible for Cl<sup>-</sup> efflux) increases while that of the Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> cotransporter-1 (responsible for Cl<sup>-</sup> influx) falls in hPSC<sup>C</sup> neurons which is in agreement with native developmental mechanisms that regulates intracellular Cl<sup>-</sup> activity (Ben-Ari *et al.*, 2007). In agreement with our data, Shcheglovitov *et al.*, 2013 also report that application of GABA to hPSC<sup>C</sup> neurons generates hyperpolarising currents consistent with a reduction in intracellular chloride activity. The time of this switch has been reported with high variability in the human cortex (Blaesse *et al.*, 2009), though it is interesting that a population of Pax-6<sup>+</sup> neural precursors give rise to subplate neurons that highly express KCC2 by 16 post conception weeks (Bayatti *et al.*, 2008) and that the Livesey *et al.*, (2014) study differentiates hPSC<sup>C</sup> neurons from predominantly Pax-6<sup>+</sup> neural precursors. Our study (Livesey *et al.*, 2014) also highlights that the use of neurotrophic media supplements influences the development of expression of Cl<sup>-</sup> transporters and Cl<sup>-</sup> activity. Although neurotrophic factors are added to promote synaptogenesis and/or increase responsiveness to neurotransmitters in PSC-derived neuronal cultures (Copi *et al.*, 2005; Bardy *et al.*, 2015), neurotrophic factors have an important role in the regulation of Cl<sup>-</sup> transporter expression (Blaesse *et al.*, 2009). Beyond pharmacological considerations regarding the use of chronic neurotrophic factor media supplements (Frank *et al.*, 1996), the impact of neurotrophic factors on PSC-derived neuronal physiology needs to be carefully considered.

## SYNAPTIC AND NETWORK PROPERTIES

In addition to harbouring excitable membranes, it is a defining feature of neurons to receive and generate synaptic signals. These exist in two general forms; phasic and tonic, and are both essential to the normal function of the CNS. The intricate and specific synaptic connectivity displayed by native cortical neurons is key to cortical network development and function (Spitzer, 2006). It is therefore critical to the development of *in vitro* hPSC<sup>C</sup>-derived neurons to recapitulate native synaptic properties. The co-localisation of pre- and post-synaptic membrane associated scaffold proteins such as synaptophysin and PSD-95, respectively, provide an indication of architectural synapse formation, but not functionality. Functional synaptic activity is generally detectable in standard cultures of *in vitro* hPSC<sup>C</sup>-derived neurons where phasic ionotropic glutamatergic receptor- and GABA<sub>A</sub>R-mediated activity has been observed. The latter property is determined by the culture protocol employed and its potential to generate GABA-ergic interneurons. However, it is largely accepted that many standard *in vitro* hPSC protocols do not generate cultures that exhibit robust synaptic activity (Bardy *et al.*, 2015). Studies examining synaptic properties therefore employ techniques to promote synaptic formation in their cultures.

Moreover, many groups now co-culture hPSC<sup>C</sup> neurons with primary rodent astrocytes that also promotes synaptic function (Johnson *et al.*, 2007) (Kim *et al.*, 2011) (Shcheglovitov *et al.*, 2013) (Wen *et al.*, 2014) (Pak *et al.*, 2015). The analysis of the field-evoked post-synaptic events in co-cultured neurons demonstrates that glutamate activates both fast AMPAR-mediated and slower GluN1/GluN2B-like NMDAR-mediated events in control neurons (Shcheglovitov *et al.*, 2013). Interestingly in this study, cell lines lacking the post-synaptic density protein SHANK3, which is reduced in expression in a neurodevelopmental disorder (Phelan-McDermid syndrome), exhibit evoked NMDAR-mediated post-synaptic currents that have faster decay kinetics, which may be consistent with an NMDAR population expressing both GluN2A and GluN2B NMDAR subunits. Alternatively, pre-synaptic neurotransmitter-release dysfunction has been modelled in hPSC<sup>C</sup> neurons derived from schizophrenia (mutant DISC1; (Wen *et al.*, 2014)) and autism (mutant NRXN1; (Pak *et al.*, 2015)) patients. Recently, Bardy *et al.*, 2015, reported that relatively sparse synaptic activity observed in PSC-derived neurons is due, in part, to the media in which the neurons are maintained. Their study utilized a culture media that promoted synapse formation and concomitantly increased functional AMPA-mediated synaptic activity in PSC-derived neurons co-cultured with mouse astrocytes. Thus, hPSC<sup>C</sup> neurons can recapitulate synaptic activity and offer the exciting potential to study synaptic dysfunction to elucidate disease



mechanisms. Table 1 describes reports of advanced culture techniques in order to study synaptic physiology.

Similarly in *in vitro* cultures, reports of robust network activity have not yet been reported widely. A potential factor in the inability to observe synchronous multi-neuronal firing in many cultures may be a consequence of the extent to which GABA-ergic interneurons are present or absent in cultures. GABA-ergic signalling is established before that of glutamatergic-signalling in early development and is thought to initiate primitive neural network activity (Ben-Ari *et al.*, 2007). Indeed, inhibitory GABA-ergic interneurons are essential for maintaining the balanced activity of cortical neural circuits. GABA-ergic synaptic activity has been detected within hPSC<sup>C</sup> cultures in the form of spontaneous post-synaptic currents (Shcheglovitov *et al.*, 2013; Johnson *et al.*, 2007; Wu *et al.*, 2007). However, a recent report has observed synchronized excitatory neurotransmitter-driven network activity that resembles that of early-stage cortical development and which occurs in cultures which contains a low percentage of interneurons and is insensitive to pharmacological blockade of GABA<sub>A</sub>Rs (Kirwan *et al.*, 2015). A key study in the near future will therefore be the culture of defined mixtures hPSC<sup>C</sup> neurons and defined populations of GABA-ergic interneurons. A key study in the near future will therefore be the culture of defined mixtures hPSC<sup>C</sup> neurons and defined populations of GABA-ergic interneurons. Notably, it has been shown that hPSC<sup>C</sup> neurons can integrate into the network firing properties generated by primary mouse cells (Weick *et al.*, 2011) and, furthermore light-stimulated hPSC-derived neurons transduced with channelrhodopsin can influence the network activity of hippocampal organotypic slices (Piña-Crespo *et al.*, 2012).

## FUTURE CHALLENGES: MAKING MATURE NEURONS

The capacity of hPSC<sup>C</sup> neurons to become physiological relevant is their successful incorporation into a native system. As an initial step along this road Espuny-Camacho *et al.*, (2013) successfully demonstrated the differentiation and functional integration of hPSC<sup>C</sup> neurons into rodent cortex. Several months after transplantation into the developing cortex hPSC<sup>C</sup> neurons exhibited intrinsic membrane properties consistent with adult mature neurons, in contrast to the more immature properties of *in vitro* differentiated hPSC<sup>C</sup> neurons. hPSC<sup>C</sup> neurons incorporated into the rodent cortex also received synaptic inputs. Integration of hPSC<sup>C</sup> neurons expressing channelrhodopsin to the mouse hippocampal CA1 region has demonstrated the ability to generate light-evoked post-synaptic currents in

adjacent (untransfected) neurons (Weick *et al.*, 2011). These data indicate the potential of such neurons to contribute to cortical network activity. Furthermore, it has been reported that hPSC<sup>C</sup> neurons integrated into a rat-model of stroke promotes functional recovery (Tornerio *et al.*, 2013). While there is clearly still much to learn with regard to *in vivo* functional integration, studies such as these give strong support to the notion that hPSC<sup>C</sup> neurons have the capacity to possess functionally mature phenotypes. Similarly, hPSC<sup>C</sup>-derived ventral telencephalic interneurons develop mature intrinsic properties and receive synaptic input when integrated into the embryonic rodent cortex (Nicholas *et al.*, 2013). Furthermore, this study indicates that interneuron maturation can be achieved with co-culture in the presence of rodent glia and requires extended culture periods (>6 months). An equivalent study hasn't been performed for hPSC<sup>C</sup> excitatory neurons. These data indicate that *in vitro* hPSC-derived neuron cultures lack important factors that are present *in vivo* and which are required for maturation.

Aside from their electrophysiological phenotype, hPSC<sup>C</sup> neurons need to display morphological characteristics typical of native cortical neurons. Dendritic spines form the principal location at which excitatory synaptic transmission and synaptic plasticity takes place and moreover, numerous neurological diseases are accompanied by spine number or size alterations (Bourne & Harris, 2008; Penzes *et al.*, 2011). In this respect it is of significance that *in vivo* integrated hPSC<sup>C</sup> neurons do appear to develop spine-like structures (Espuny-Camacho *et al.*, 2013), but dendritic spines structures with co-localised expression of PSD-95 are infrequent in *in vitro* hPSC-derived neurons (Marchetto *et al.*, 2010).

In conclusion, a major challenge is to generate neuronal populations that exhibit maturation profiles that more closely reflect those seen *in vivo*. Co-culture with astrocytes and mixed neuronal populations together with the maintenance of cells in media that promotes increased synaptic activity indicates that such strategies are required to assess synaptic transmission. When achieved, this will enhance and make more relevant our ability to study of human physiology and pathophysiology using *in vitro* hPSC<sup>C</sup> neurons.

## COMPETING INTERESTS

The authors declare no conflict of interests.

## AUTHOR CONTRIBUTIONS

All authors contributed to design and writing of manuscript.

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**Table 1.** Advanced culture techniques using hPSC<sup>C</sup> neurons (or other hPSC-derived neurons) that promote functional synaptic formation and/or maturation.

Approach	Study	Advanced protocol detail	Physiological detail measured from hPSC <sup>C</sup> neurons
Astrocyte co-culture	Johnson <i>et al.</i> , 2007	Co-culture with primary E14 mouse astrocytes.	Accelerated initial functional synapse formation, but long-term unaffected. Spontaneous post-synaptic currents blocked by AMPAR and GABA <sub>A</sub> R antagonists
	Shcheglovitov <i>et al.</i> , 2013	Co-culture with primary rat cortical astrocytes.	Spontaneous and evoked post-synaptic currents that consist of AMPAR, GABA <sub>A</sub> R and NMDAR components. Hyperpolarising GABA <sub>A</sub> R responses. High $R_{IN}$ .
	Wen <i>et al.</i> , 2014	Co-culture with primary rat astrocytes	Spontaneous post-synaptic currents. Neurotransmitter release investigated using FM1-43 imaging.
	Pak <i>et al.</i> , 2015	Co-culture with primary mouse astrocytes.	Evoked AMPAR-mediated post-synaptic currents.

High  $R_{IN}$ .

<b>Neuron co-culture</b>	Kim <i>et al.</i> , 2011	Co-culture with primary E18 rat cortical neurons (or with rat astrocytes).	Spontaneous post-synaptic currents. High $R_{IN}$ , as indicated by low rheobase needed to elicit action potential firing.
	Weick <i>et al.</i> , 2011	Co-culture with primary E16 rat cortical neurons.	Neurons adopt bursting activity of mouse neurons. Light-activation of channelrhodopsin transduced neurons induces AMPAR sensitive bursting in mouse neurons.
<b>Advanced media composition + astrocyte co-culture</b>	Bardy <i>et al.</i> , 2015	Custom media formulation including media supplements. hPSC-derived neurons co-cultured with primary mouse astrocytes.	Neurons maintained in new formulation exhibit increased frequency of AMPAR-mediated post-synaptic currents, but not GABA <sub>A</sub> R-mediated post-synaptic currents. High $R_{IN}$ .
<b>3-dimensional culture</b>	Lancaster <i>et al.</i> , 2013	'Cerebral organoid' development	TTX-sensitive spontaneous activity detected using $Ca^{2+}$ -imaging. Increase in $Ca^{2+}$ detection upon application of glutamate.
	Pasca <i>et al.</i> , 2015	'Cortical spheroid' development equivalent to 19-24 weeks foetal development.	Spontaneous firing activity and evoked excitatory post-synaptic currents blocked by glutamate receptor antagonists. High $R_{IN}$ , as indicated by low rheobase needed to elicit action potential firing.
<b>Integration</b>	Weick <i>et al.</i> , 2011	Integration of hPSC <sup>C</sup> neurons into mouse (aged 2 months) hippocampus.	Light-activation of channelrhodopsin transduced neurons induces synaptic events in adjacent mouse neurons.
	Espuny-Camacho <i>et al.</i> , 2013	Integration of hPSC <sup>C</sup> neurons into embryonic mouse cortex. Assessed 9 months post-integration after key	Spontaneous firing activity and evoked post-synaptic currents that can be blocked by glutamate receptor antagonists. Low $R_{IN}$ .

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developmental period.

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